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Bcl-2 and Bcl-X_L Regulate Proinflammatory Caspase-1 Activation by Interaction with NALP1

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SUMMARY

Caspases are intracellular proteases that cleave substrates involved in apoptosis or inflammation. In *C. elegans*, a paradigm for caspase regulation exists in which caspase CED-3 is activated by nucleotide-binding protein CED-4, which is suppressed by Bcl-2-family protein CED-9. We have identified a mammalian analog of this caspase-regulatory system in the NLR-family protein NALP1, a nucleotide-dependent activator of cytokine-processing protease caspase-1, which responds to bacterial ligand muramyl-dipeptide (MDP). Antiapoptotic proteins Bcl-2 and Bcl-X_L bind and suppress NALP1, reducing caspase-1 activation and interleukin-1β (IL-1β) production. When exposed to MDP, Bcl-2-deficient macrophages exhibit more caspase-1 processing and IL-1β production, whereas Bcl-2-overexpressing macrophages demonstrate less caspase-1 processing and IL-1β production. The findings reveal an interaction of host defense and apoptosis machinery.

INTRODUCTION

Caspases are intracellular proteases that cleave substrates involved in either apoptosis or inflammation, with different branches of the caspase family devoted to these two functions in mammals. The zymogen forms of all inflammatory, and some apoptotic, caspases contains an N-terminal CARD domain that mediates their interactions with various adaptor proteins, thereby controlling their activation, often through a mechanism involving oligomerization (reviewed in (Martinon and Tschopp, 2004). In *Caenorhabditis elegans* (*C. elegans*), a paradigm for apoptotic caspase regulation has been established in which the CARD-containing caspase CED-3 is activated by CED-4, a nucleotide-binding, CARD-containing protein that oligomerizes to create a platform for protease activation (Riedl et al., 2005). CED-4 is directly suppressed by Bcl-2-family member CED-9, an antiapoptotic protein that binds CED-4 (Metzstein et al., 1998). Given the similarities in apoptosis mechanisms throughout the animal kingdom, it has been hypothesized that mammalian Bcl-2-family proteins also directly regulate caspase activators, but no convincing examples have heretofore been revealed.

NLR-family proteins (also called NALPs, NODs, CATERPILLER, or PAN) constitute a large family of caspase-activating and NF-κB-activating proteins found in vertebrates and in marine vertebrates but not *C. elegans* or *Drosophila*. These proteins uniformly contain a putative nucleotide-binding fold called NACHT, plus leucine-rich repeat (LRR) domains, typically in combination with additional protein-interaction domains, including PYRIN and CARD domains (reviewed in Kufer et al., 2005; Martinon and Tschopp, 2005; Stehlik and Reed, 2004; Ting et al., 2006). The NACHT domain mediates oligomerization of mammalian NLRs, analogous to the nucleotide-binding NB-ARC domain of CED-4 in *C. elegans*. Evidence has been presented to suggest that the LRRs suppress NACHT-mediated oligomerization, with this repression relieved upon binding microbial ligands (Martinon et al., 2002; Poyet et al., 2001). In this regard, NLRs represent the intracellular complement to the cell-surface TLR-family receptors involved in innate immunity in animals and are highly analogous to intracellular host-defense proteins of plants (Kufer et al., 2005; Martinon and Tschopp, 2005; Stehlik and Reed, 2004).

Here we show that the human NLR-family member NALP1 (NAC/CARD7/DEFCAP/CLR17.1/NLRP1) is regulated by interactions with antiapoptotic proteins Bcl-2 and Bcl-X_L, which suppress NALP1-mediated activation of caspase-1 and reduce production of the caspase-1 substrate interleukin-1β. NALP1 is similar to CED-4 in that it contains CARD- and nucleotide-binding oligomerization domains. The Bcl-2/Bcl-X_L-mediated suppression of caspase-1-activating NALP1 thus provides a mammalian analog to the *C. elegans* system and reveals a novel mechanism linking host defense and apoptosis.

RESULTS

NALP1 Binds Bcl-2 and Bcl-X_L

We surveyed members of the NALP family for interactions with antiapoptotic human Bcl-2-family proteins. NALP1 was found to associate with Bcl-2 and Bcl-X_L by coimmunoprecipitation (coIP) experiments using lysates prepared from transfected HEK293T cells expressing epitope-tagged proteins. Of the six human antiapoptotic Bcl-2-family proteins, only Bcl-2 and Bcl-X_L associated with NALP1. In contrast, Mcl-1, Bcl-W, Bfl-1, and Bcl-B did not associate with NALP1 (Figures 1A and 1B), nor did various proapoptotic Bcl-2-family proteins, including Bax, Bak, Bid, and Bcl-G (Figure 1B). Similar conclusions were reached using in vitro protein-binding assays where NALP1-containing cell lysates were incubated with bacteria-produced GST-fusion proteins (Figure 1C).

To explore whether NALP1 is unique among NLR-family proteins in its ability to bind Bcl-2 and Bcl-X_L, we compared NALP1 with NALP2, -3, and -4, which all contain PYRIN, NACHT, and LRR domains like NALP1. We also examined the proteins Pyrin and ASC, which contain PYRIN domains. However, among these proteins tested, only NALP1 associated with Bcl-X_L and Bcl-2 (Figures 1D and 1E; data not shown).

NALP1 forms a multiprotein caspase-activating complex called the ''inflammasome,'' which contains NALP1, bipartite-adaptor protein ASC (containing PYRIN and CARD domains), and caspase-1 (Martinon et al., 2002). Both lipopolysaccharide (LPS) and the peptidoglycan component muramyldipeptide (MDP) have been reported to stimulate NALP1 inflammasome assembly (Faustin et al., 2007; Martinon et al., 2002). To explore the interaction of endogenous Bcl-2 and Bcl-X_L with endogenous NALP1, we performed experiments with THP-1 monocytes that had been differentiated into macrophages using phorbol ester TPA and followed procedures that were previously published in which treatment of these cells with either LPS or MDP (along with ATP to enhance IL-1β release) was shown to induce inflammasome assembly, caspase-1 activation, and IL-1β secretion (Martinon et al., 2002). Treatment of macrophages with LPS or MDP did not significantly alter total cellular levels of NALP1, ASC, Bcl-2, Bcl-X_L, or procaspase-1 as determined by immunoblotting (Figures 2A–2D; data not shown), but it did stimulate IL-1β secretion (Figure 2A). When endogenous NALP1 was immunoprecipitated from untreated macrophages using anti-NALP1 antibody, endogenous Bcl-2 and Bcl-X_L were associated with NALP1-containing immune complexes, while ASC was not (Figure 2B). These macrophages evidently contain more Bcl-2 than Bcl-X_L, which possibly accounts for the clearer association of Bcl-2 with NALP1 immunoprecipitates when compared to Bcl-X_L. In contrast, when immunoprecipitated from MDP/ATP-treated (Figure 2B) or LPS/ATP-treated (Figure S1) macrophages, ASC was associated with NALP1-containing immune complexes, while Bcl-2 and Bcl-X_L were not. These findings were confirmed by reciprocal coIP experiments using anti-Bcl-2 (Figure 2C), anti-Bcl-X_L (not shown), or anti-ASC (Figure 2D) antibodies. Subcellular fractionation studies showed that these LPS/ATP-inducible differences in NALP1 binding to ASC and Bcl-2 also correlated with changes in the relative amounts of NALP1 associated with membranous organelles where Bcl-2 is located (Figure 2E).

Bcl-2 and Bcl-X_L Suppress Caspase-1 Activation by NALP1

The NALP1 inflammasome binds caspase-family proteases involved in proteolytic processing of proinflammatory cytokine prointerleukin-1β (IL-1β), including procaspase-1 and procaspase-5, but not caspase-9 or caspase-12 (Figure S2). We therefore explored the effect of overexpressing antiapoptotic Bcl-2-family proteins on NALP1-induced production of IL-1β. When 293 cells were transfected with plasmids encoding the inflammasome components NALP1, ASC, and procaspase-1 as well as the inflammasome substrate pro-IL-1β, we observed mature IL-1β secretion into culture medium (detected by ELISA) and production of 17 kDa cleaved IL-1β protein in cells (detected by immunoblotting; Figure 3A). Cotransfection of Bcl-2 or Bcl-X_L markedly suppressed NALP1-dependent IL-1β secretion as well as production of intracellular cleaved p17 IL-1β. Immunoblotting experiments showed that Bcl-2 and Bcl-X_L did not alter the levels of the various inflammasome components (not shown). In contrast to Bcl-2 and Bcl-X_L, antiapoptotic Bcl-2-family proteins that do not bind NALP1 do not suppress IL-1β secretion or pro-IL-1β cleavage; these include Bcl-W, Bcl-B, Bfl-1, and Mcl-1. Moreover, none of the six antiapoptotic Bcl-2-family proteins modulated IL-1β production induced by transfection of cells with procaspase-1 alone or in combination with an alternative NLR-family protein (NALP2/PAN1) that does not bind Bcl-2-family proteins (Figure S3), thus confirming the specificity of these results. However, all six antiapoptotic human Bcl-2-family proteins effectively suppressed apoptosis and reduced activation of apoptotic caspases when expressed in 293 cells by the same transfection method (Figure S3B), confirming the bioactivity of these proteins. Similar results regarding Bcl-2 and Bcl-X_L suppression of NALP1-induced IL-1β production were obtained using HeLa cells (not shown) except that transfection of ASC was not required because these cells express ASC endogenously (Figure S4).

We attempted to reconstitute in vitro the NALP1-dependent activation of procaspase-1 so that the effects of Bcl-X_L and Bcl-2 could be tested directly and modeled our approach after previously described cell-free systems for studying NALP1-mediated activation of caspase-1 (Martinon et al., 2002). Extracts from THP-1 macrophages were mixed with extracts from NALP1-transfected 293T cells and then incubated at 37°C to induce caspase-1 activation in the presence or absence of recombinant Bcl-2-family proteins. Adding Bcl-2 or Bcl-X_L to extracts suppressed caspase-1 activity as measured by hydrolysis of

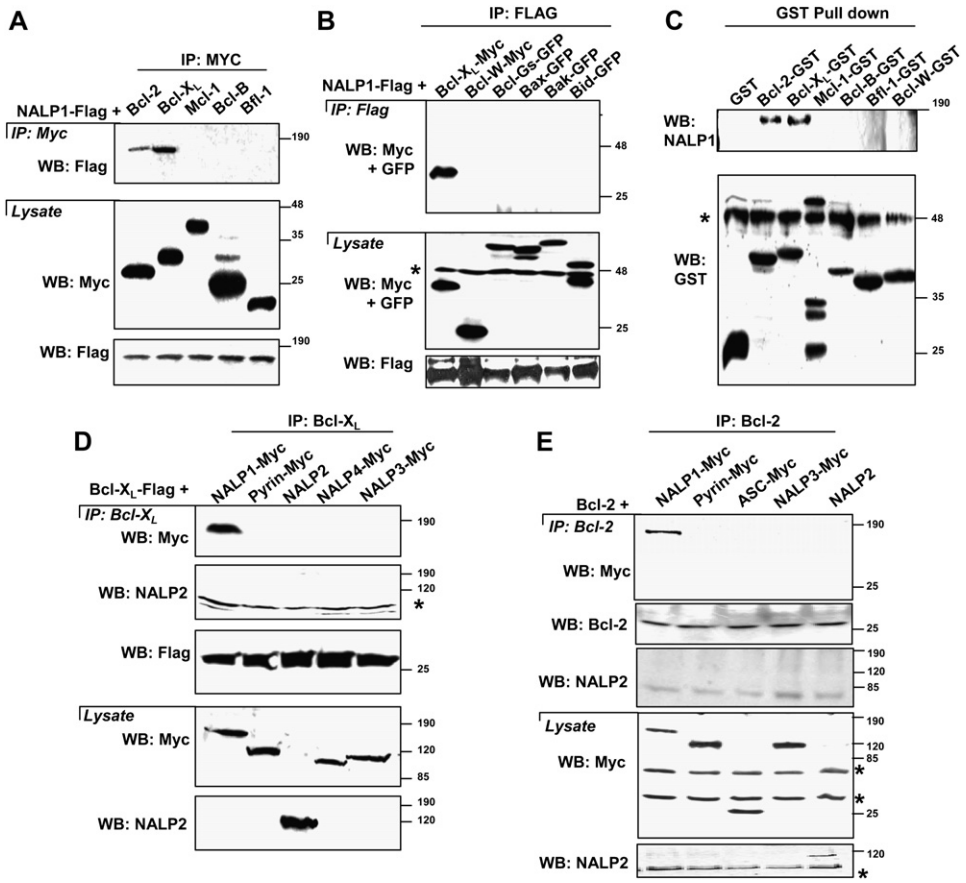

Figure 1. Bcl-2 and Bcl-X_L Bind NALP1

(A) and (B) show CoIP assays. HEK293T cells were transfected with plasmids encoding Flag-NALP1 and either Myc-tagged Bcl-2, Bcl-X_L, Mcl-1, Bcl-B, Bfl-1 (A), Bcl-X_L, or Bcl-W proteins and GFP-tagged Bcl-Gs, Bax, Bak and Bid (B). Cell lysates normalized for total protein content were analyzed directly (middle and bottom) or subjected to IP (top) using anti-Myc (A) or anti-Flag (B) antibody and were analyzed by SDS-PAGE/immunoblotting (WB) using anti-Flag (A), anti-Myc, and anti-GFP (B) antibodies.

(C) GST pull-down assays are shown. Cell extract from 293T cells overexpressing NALP1 (20 μg per sample) was incubated with 1 μg of immobilized GST or GST-fusion proteins corresponding to various Bcl-2-family proteins. Proteins associated with glutathione-Sepharose were analyzed by immunoblotting using anti-GST (bottom) and anti-NALP1 (top) antibodies.

(D and E) Bcl-X_L and Bcl-2 uniquely bind NALP1 among the NLR-family proteins that were tested. Lysates were prepared from 293T cotransfected with plasmids encoding Flag-Bcl-X_L (D) or Bcl-2 (E) and plasmids encoding Myc-tagged NALP1, NALP3, NALP4, Pyrin, ASC, or untagged NALP2. Lysates were analyzed directly (bottom) or subjected to IP using anti-Bcl-X_L or anti-Bcl-2 antibody (top), then analyzed by immunoblotting using anti-Flag, anti-Myc, or anti-NALP2 antibodies, as indicated. All results are representative of at least three independent experiments. Asterisks denote nonspecific bands. Molecular weight (MW) markers are indicated in kilodaltons.

fluorogenic substrate acetyl-Tryptophanyl-Glutamyl-Histindinyl-Aspartyl-aminofluorocoumarin (Ac-WEHD-AFC; Figure 3B). In contrast, Bcl-W, Bfl-1, Bcl-B, or Mcl-1 did not significantly suppress NALP1-dependent caspase-1 activation in extracts. Also, when THP-1 macrophages were pretreated with LPS to induce activation of caspase-1 prior to preparing extracts, then Bcl-2 (Figure S5) and Bcl-X_L (not shown) failed to suppress caspase-1 activity in vitro, showing that Bcl-2 and Bcl-X_L do not suppress caspase-1 after it has become activated.

NALP1-containing extracts were also used for interrogating mechanisms by which Bcl-X_L suppresses NALP1 activation. We used NALP1 ligand MDP instead of LPS because of its superior potency (Faustin et al., 2007). Note that commercial preparations of LPS are typically contaminated with MDP-containing peptidoglycan, which may account for their ability to activate NALP1. For these experiments, the bacterial form of MDP was compared with an inactive enantiomer, MDP-DD. Prior to MDP exposure, the caspase-1-binding adaptor ASC is not associated with NALP1 (Figures S6 and 3C). When active MDP-LD (but not MDP-DD) was added to extracts derived from HEK293T cells transfected with plasmids encoding GFP-tagged ASC and epitope-tagged NALP1, we observed that GFP-ASC inducibly associated with NALP1 (Figures 3C and 3D). Addition of Bcl-X_L or Bcl-2 to the extracts prevented GFP-ASC from binding to NALP1. Thus, Bcl-X_L and Bcl-2 prevent inflammasome formation in vitro

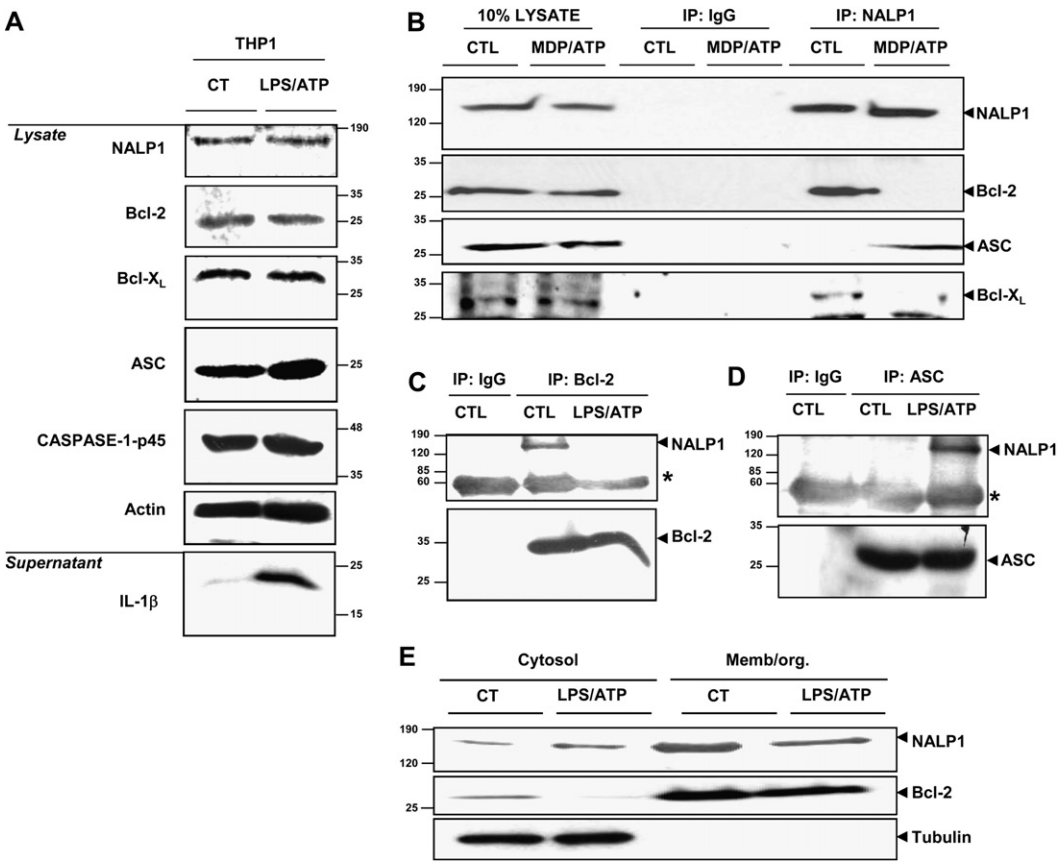

Figure 2. Endogenous NALP1 Binds Bcl-2

(A) TPA-differentiated THP-1 cells were cultured with or without (CT) 1 μg/ml crude LPS for 20 hr and then pulsed with 5 mM ATP for 10 min. Cell lysates were normalized for protein content and analyzed by immunoblotting using antibodies specific for NALP1, Bcl-2, Bcl-X_L, and ASC. Culture supernatants (normalized for volume) were also analyzed for IL-1β by immunoblot analysis.

(B) THP-1 macrophages were cultured with or without (CTL) MDP/ATP as above, and lysates were prepared for IP with control IgG or anti-NALP1 antibody. The resulting immune complexes were analyzed by immunoblotting using antibodies recognizing NALP1 (top), Bcl-2, ASC, or Bcl-X_L (bottom). Ten percent of lysates were also loaded directly as a control (left lanes).

(C and D) THP-1 macrophages were prepared as above, and lysates were subjected to IP using control (CTL) IgG and either anti-Bcl-2 (C) or anti-ASC (D) antibodies. Immune complexes were fractionated by SDS-PAGE (8% gels, C; 14% gels, D) and analyzed by immunoblotting using anti-NALP1 (top) antibody followed by anti-Bcl-2 or anti-ASC (bottom).

(E) LPS changes subcellular location of NALP1. THP-1 macrophages were treated with or without 5 μg/ml crude LPS for 4 hr and 2.5 mM ATP for 10 min, then cytosolic and membrane fractions were prepared and analyzed by immunoblotting using anti-NALP1, -Bcl-2, or -tubulin antibodies. All results are representative of ≥3 independent experiments. Asterisks denote nonspecific bands.

at least in part by blocking ASC recruitment to NALP1 after MDP stimulation. Control proteins, such as GST-Bcl-B, which does not bind NALP1, did not have this effect (Figure 3D; data not shown). We hypothesize, therefore, that Bcl-2 and Bcl-X_L recognize an inactive conformation of NALP1 and suppress conversion of NALP1 to the active conformation that binds ASC and allows inflammasome assembly.

Binding Is Required for Suppression of NALP1 by Bcl-2 and Bcl-X_L

Domain-mapping experiments were performed to explore whether binding is required for Bcl-2 and Bcl-X_L to suppress NALP1-induced activation of caspase-1 and production of IL-1β. Antiapoptotic Bcl-2-family proteins contain conserved BH1-4 domains and are homologous throughout their amino acid sequences with the exception of a loop of variable length between BH4 (α-helix-1) and BH3 (α-helix-2; Strasser, 2005). To explore why Bcl-2 and Bcl-X_L uniquely bind NALP1 among the six antiapoptotic Bcl-2 family members, we compared full-length Bcl-2 and Bcl-X_L with various deletion mutants. Removal of the loop from Bcl-2 or Bcl-X_L abolished interaction with NALP1 (Figures 4A and S7A). In contrast, deleting BH3 or BH4 domains from Bcl-X_L did not impair binding to NALP1, as determined by coIP experiments (Figure S7B). These protein-interaction studies were performed by coIP using cell lysates and were independently confirmed

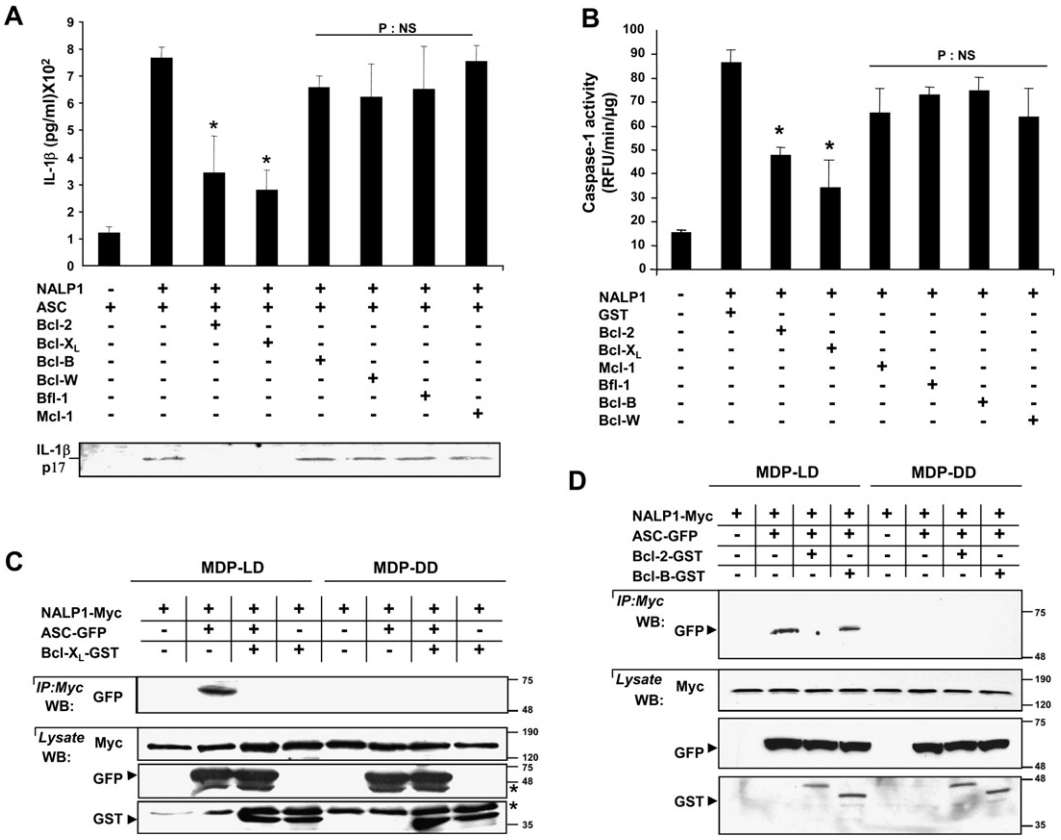

Figure 3. Bcl-2 and Bcl-X_L Suppress Caspase-1 Activation by NALP1

(A) Bcl-2 and Bcl-X_L suppress NALP1-induced IL-1β production. 293 cells were transfected with plasmids encoding procaspase-1, pro-IL-1β, and ASC with (+) or without (−) NALP1 according to established procedures (Martinon et al., 2002) and with various plasmids encoding antiapoptotic Bcl-2-family proteins as indicated, with total DNA constant maintained at 1 μg by addition of empty plasmid. Supernatants were analyzed by ELISA for IL-1β secretion at 24 hr posttransfection (mean ± SD; n = 3; *top*). Asterisk indicates $p < 0.05$. Lysates were normalized for protein content and analyzed by immunoblotting (WB) using anti-IL-1β antibody (bottom).

(B) Bcl-2 and Bcl-X_L suppress NALP1-mediated caspase-1 activation in vitro. THP-1 cells were differentiated into macrophages by overnight treatment with 50 ng/ml TPA, and cytosolic extracts were prepared. Extracts from 293T cells transfected with NALP1-encoding plasmid were mixed 2:1 with THP-1 extracts and incubated with 5 μg GST protein or various GST-fusion proteins corresponding to antiapoptotic Bcl-2-family proteins for 45 min at 37°C. Caspase-1 activity was measured using Ac-WEHD-AFC substrate, measuring relative fluorescence units (RFU) of AFC released per min per mg of protein (mean ± SD; n = 3). Asterisk indicates $p < 0.05$. All experiments were repeated at least three times.

(C and D) Bcl-X_L and Bcl-2 inhibit MDP-induced association of NALP1 with ASC in vitro. Extracts (20 μg) from 293T cells transfected with a normalized amount of DNA, including plasmids encoding Myc-NALP1 and GFP-ASC, were incubated with or without GST-Bcl-X_L, GST-Bcl-2, or GST-Bcl-B, then MDP-LD or MDP-DD was added to stimulate inflammasome assembly. IPs were performed using anti-myc antibody followed by SDS-PAGE/immunoblot analysis using anti-GFP (top). Alternatively, lysates were directly analyzed by immunoblotting (WB) using anti-myc, -GFP, or -GST antibodies (bottom). All results are representative of ≥3 independent experiments. Asterisks denote nonspecific bands, while arrowheads indicate specific bands.

by immunofluorescence confocal microscopy analysis of intact cells, where full-length Bcl-2, but not Bcl-2(Δloop), was shown to cause redistribution of NALP1 from a diffuse cytosolic to an organellar location (Figure S8). Correlating with the protein interaction, mutants of Bcl-X_L (Figure 4B) or Bcl-2 (Figure 4C) that lacked the loop were also inactive with respect to suppression of NALP1-induced IL-1β secretion and NALP1-induced proteolytic processing of intracellular pro-IL-1β. Because Bcl-X_L(Δloop) and Bcl-2(Δloop) mutants have enhanced antiapoptotic activity (Chang et al., 1997), NALP1-suppressing activity can be separated from antiapoptotic activity of Bcl-X_L and Bcl-2. Similarly, a point mutant of Bcl-2 (G145A) lacking antiapoptotic activity (Yin et al., 1994) retained NALP1-binding activity (data not shown) and significantly inhibited NALP1-induced IL-1β production, again dissociating NALP1-suppressing activity from apoptosis-suppressing activity (Figure 4C).

Using a series of truncation and internal deletion mutants of NALP1, we attempted to map the region of NALP1 required for binding Bcl-X_L. These experiments demonstrated that the LRRs (but not the CARD or FIIND domains)

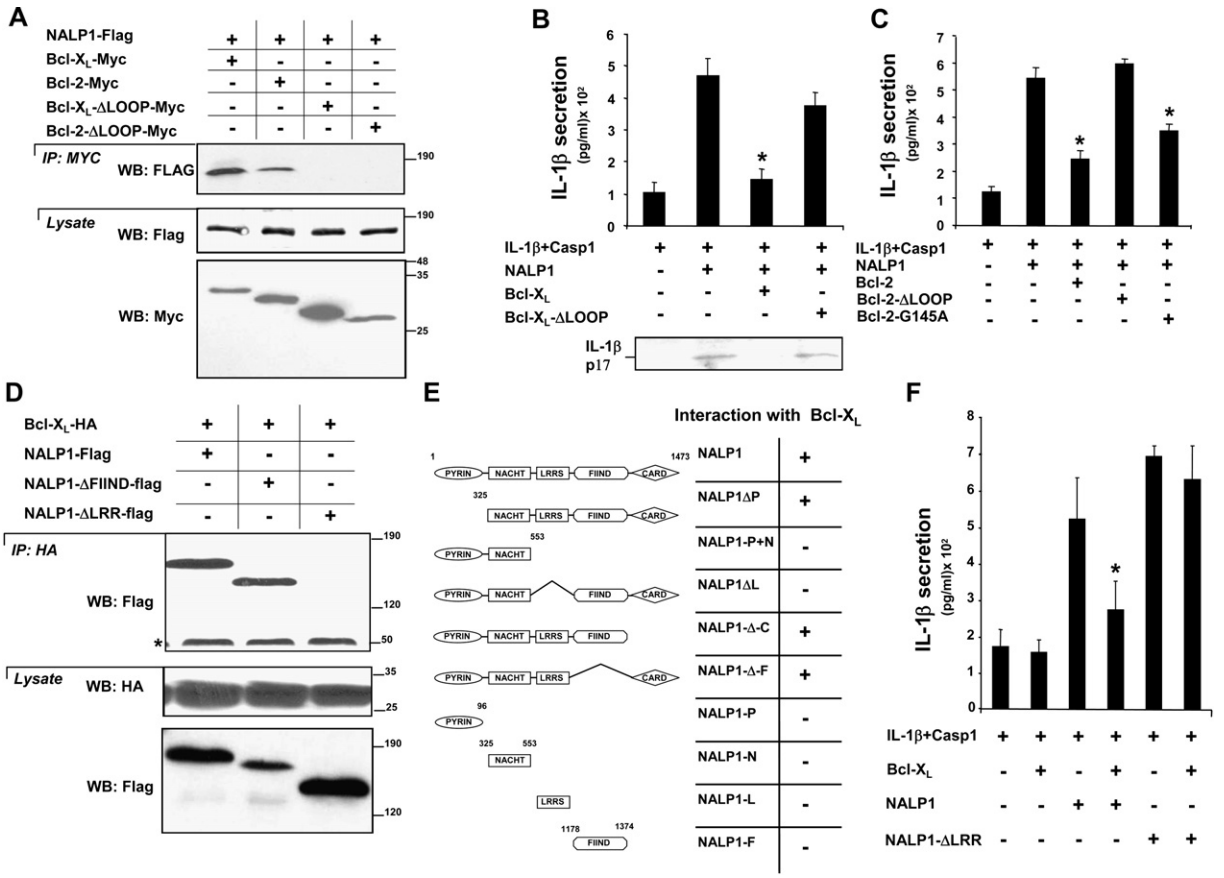

Figure 4. Binding Is Required for Suppression of NALP1-Induced IL-1β Production by Bcl-2 and Bcl-X_L

(A and B) Loops of Bcl-X_L and Bcl-2 required for NALP1 binding are shown. 293T cells were transfected with Flag-NALP1 plasmid in combination with plasmids encoding Myc-Bcl-2, Myc-Bcl-2-ΔLOOP, Myc-Bcl-X_L, or Myc-Bcl-X_L-ΔLOOP. Lysates were either analyzed directly (10 μg protein; bottom) or subjected to IP using anti-Flag (top) and analyzed by immunoblotting using anti-Myc antibody.

(B and C) Interaction of NALP1 with Bcl-X_L is required for inhibition of NALP1-induced IL-1β production. HeLa cells were transfected with plasmids encoding NALP1, procaspase-1, and pro-IL-1β in combination with plasmid encoding Bcl-X_L versus Bcl-X_LΔloop (B) or Bcl-2 versus Bcl-2Δloop (C), with IL-1β production measured at 24 hr. Additionally, wild-type Bcl-2 and Bcl-2-G145A were compared (C), which further dissociated suppression of NALP1-induced IL-1β production from antiapoptotic activity. In (B), cell lysates were normalized for protein content and analyzed by immunoblotting using anti-IL-1β antibody (bottom).

(D and E) LRR of NALP1 is necessary but insufficient for binding Bcl-X_L. Interactions of Bcl-X_L with full-length NALP1 and various NALP1-deletion mutants were tested by coIP assay. Representative data are shown for ΔLRR and ΔFIIND mutants. (E) shows summary of results (see Figures S7C and S7D).

(F) NALP1ΔLRR mutant is resistant to suppression by Bcl-X_L. HeLa cells were transfected as above, by comparing full-length NALP1 with NALP1ΔLRR and measuring IL-1β production in the presence or absence of cotransfected Bcl-X_L. Supernatants in panels (B), (C), and (E) were analyzed by ELISA for IL-1β at 24 hr posttransfection (mean ± SD; n = 3). Asterisk indicates $p < 0.05$ compared to NALP1.

of NALP1 are necessary, but insufficient, for binding Bcl-X_L (Figures 4D, 4E, S7C, and S7D). These protein-interaction studies were performed by coIP using cell lysates and were independently confirmed by immunofluorescence confocal microscopy analysis of intact cells, where full-length NALP1 but not NALP1ΔLRR was shown to redistribute from a diffuse cytosolic to an organellar location when coexpressed with Bcl-2 (Figure S8). Consistent with the protein-interaction data showing that the LRRs of NALP1 are required for binding Bcl-X_L, we observed that IL-1β production induced by a mutant of NALP1 lacking the LRRs was not suppressed by Bcl-X_L, in contrast to full-length NALP1 (Figure 4F). We conclude, therefore, that Bcl-2 and Bcl-X_L must bind NALP1 to suppress NALP1-mediated IL-1β production.

Bcl-2 Regulates MDP-Induced IL-1β Production in Macrophages

We experimentally manipulated the levels of Bcl-2 or Bcl-X_L in human THP-1 macrophages using RNA interference (RNAi) and gene transfer then studied effects on MDP-induced IL-1β production. In cultured human THP-1 macrophages, siRNA experiments demonstrated that IL-1β production (measured by ELISA) in response to MDP is

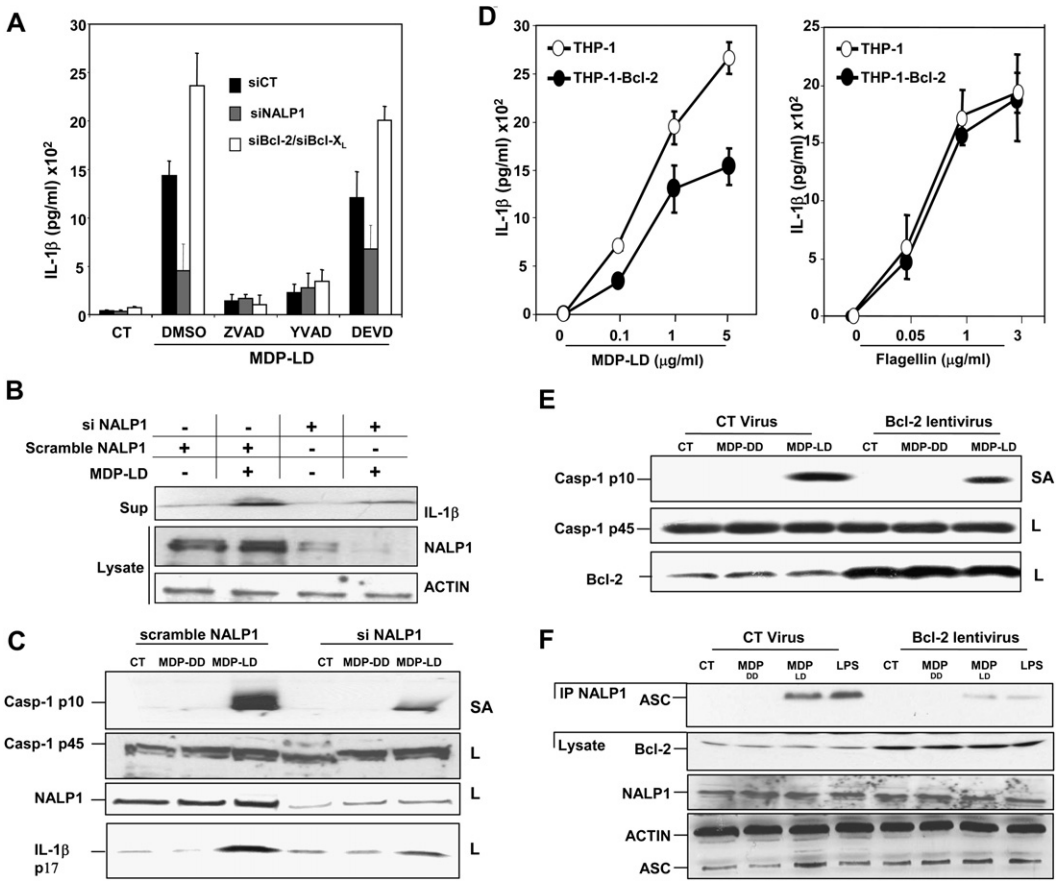

Figure 5. Bcl-2 and Bcl-X_L Regulate MDP-Inducible IL-1β Production in Macrophages

(A) Regulation of MDP-induced IL-1β production by NALP1, Bcl-2, and Bcl-X_L. THP-1 monocytes were electroporated with various siRNAs (2 μg total), including NALP1, control (CT; scrambled NALP1 sequence), or a 1:1 mixture of siRNA (1 μg each) targeting Bcl-X_L and Bcl-2. Cells were differentiated by TPA stimulation then cultured with or without 50 mM zVAD-fmk, Ac-YVAD-fmk, or Ac-DEVD-fmk and treated with MDP-LD or MDP-DD for 2 hr before pulsing with 2.5 mM ATP for 20 min. Supernatants were collected 2 hr later and IL-1β secretion was measured by ELISA (mean ± SD; n = 3).

(B) Immunoblot analysis of siRNA-transfected cells is shown. Lysates were prepared from THP-1 cells treated as above, or culture supernatants were TCA precipitated and analyzed by immunoblotting, using antibodies specific for IL-1β (supernatant; top), NALP1 (lysate; middle), or β-actin (lysates; bottom).

(C) NALP1 regulates MDP-induced caspase-1 activation. THP-1 cells were electroporated with NALP1 or scrambled siRNAs and treated as above with MDP-LD or MDP-DD for 2 hr. Cell lysates (L) were normalized for protein content and either incubated with biotinyl-VAD-fmk to capture active caspase-1 using streptavidin-Sepharose (SA; top) or analyzed directly. Immunoblotting was performed using antibodies specific for p10 fragment of active caspase-1 (top), procaspase1 (p45), NALP1, or mature IL-1β (bottom).

(D) Bcl-2 overexpression suppresses MDP-induced IL-1β production. THP-1 macrophages infected with control (white circles) or Bcl-2-encoding (black circles) recombinant lentiviruses were treated with various concentrations of either MDP (left) or bacterial flagellin (right). IL-1β production was measured 24 hr later (mean ± SD; n = 3).

(E) Bcl-2 suppresses MDP-induced caspase-1 activation. THP-1 cells were infected by control or Bcl-2-encoding lentivirus, differentiated into macrophages with TPA, and then cultured with MDP-LD or MDP-DD. Lysates were processed as in (C).

(F) Bcl-2 inhibits inflammasome assembly in macrophages. THP-1 macrophages infected with control or Bcl-2-encoding lentiviruses were unstimulated (C = control) or stimulated with MDP-DD, MDP-LD, or LPS for 6 hr. Then, lysates were used for IP (top) using anti-NALP1 antibody. Immune complexes (top) or lysates (bottom) were analyzed by SDS-PAGE/immunoblotting using antibodies recognizing ASC, Bcl-2, or NALP1.

largely NALP1-dependent (Figures 5A and S9) even though at least three NLR-family members (NALP1, Nod2, and Cryopyrin) are known to respond to this bacterial peptide (Inohara et al., 2003; Martinon et al., 2004). Moreover, MDP-induced IL-1β production by THP-1 macrophages was suppressed by chemicals that inhibit caspase-1 (Ac-YVAD-fmk and zVAD-fmk) but not by compounds that preferentially inhibit effector caspases involved in apoptosis (Ac-DEVD-fmk), consistent with involvement of inflammatory caspases (Figure 5A). Immunoblot analysis confirmed sequence-specific reduction in NALP1 protein in siRNA-treated THP-1 cells and independently verified that MDP-induced IL-1β production was suppressed (Figures 5B and S9). Moreover, NALP1-targeting siRNA (but not scrambled RNA control) greatly reduced proteolytic processing of caspase-1 and of intracellular

pro-IL-1β induced in THP-1 macrophages by MDP-LD (Figure 5C).

In THP-1 macrophages where MDP-induced IL-1β production is mostly NALP1 dependent, siRNA-mediated reductions in Bcl-2 and Bcl-X_L (Figure S10) caused an increase in MDP-stimulated IL-1β production (Figure 5A), suggesting that endogenous Bcl-2 and Bcl-X_L restrain NALP1-dependent IL-1β production. In contrast, siRNAs targeting Bcl-2-family proteins that fail to bind NALP1 (i.e., Bcl-B and Bcl-W) did not significantly impact MDP-induced IL-1β production (Figure S10). Immunoblot analysis confirmed that siRNA-treatments produced reductions in the relevant proteins (Figure S10). Some siRNA reagents targeting other Bcl-2-family members have nucleotide compositions closely approximating either the Bcl-2- or Bcl-X_L-specific siRNAs, and thus serve as controls.

While siRNA-mediated knockdown of Bcl-2 and Bcl-X_L enhanced MDP-induced IL-1β production, overexpression of Bcl-2 in THP-1 macrophages had the opposite effect (Figure 5D, left). The specificity of Bcl-2-mediated suppression of MDP-induced IL-1β production was confirmed by experiments using bacterial flagellin (Figure 5D, right), which stimulates an alternative NLR-family member (Ipaf/CLAN; Franchi et al., 2006; Miao et al., 2006) that does not bind Bcl-2 or Bcl-X_L (shown). Time course studies suggested that Bcl-2-mediated suppression of MDP-induced IL-1β production is demonstrable within 4 hr and excluded differences in macrophage survival as an explanation for the difference in IL-1β release (Figure S11). Bcl-2 overexpression in THP-1 macrophages also inhibited MDP-stimulated proteolytic processing of caspase-1 (Figure 5E). We also observed that Bcl-2 overexpression inhibited inflammasome assembly in THP-1 cells whether induced by MDP or by LPS, and less endogenous ASC coIPed with endogenous NALP1 in Bcl-2-overexpressing THP-1 macrophages (Figure 5F).

Similar conclusions were reached from studies using cultured bone marrow-derived macrophages from *bcl-2* knockout and *bcl-2* transgenic mice (Domen et al., 2000; Wang et al., 2005). Direct comparisons showed that MDP induced more IL-1β production in cultures of macrophages from *bcl-2*$^{-/-}$ mice compared to *bcl-2*$^{+/-}$ mice, which in turn produced more IL-1β than cells from *bcl-2*$^{+/+}$ mice (Figure 6A). Indeed, Bcl-2-deficient macrophages produced 33% ± 6% more IL-1β than wild-type macrophages. Conversely, macrophages from transgenic mice that overexpress Bcl-2 in blood cells that are driven by a H2K promoter (Domen et al., 2000) elaborated 37% ± 7% less IL-1β compared to control cells from nontransgenic littermates (Figure 6A). These findings are particularly striking when recognizing that MDP is capable of triggering both NALP1-dependent and NALP1-independent pathways for IL-1β production and that Bcl-2 only suppresses the NALP1-dependent contribution. Also, the absolute difference in IL-1β production under the conditions of these experiments was ~500–1000 pg/mL, which is significant in view that cultured monocytes from Muckles-Wells patients (which contain hereditary mutations in a NLR gene) show IL-1β differences of only 30–900 pg/ml compared to normal cells (Agostini et al., 2004), and levels of IL-1β in serum of septic mice reportedly average 350 pg/mL (Saito et al., 2003). In contrast to IL-1β, MDP-induced production of TNFα was not different among macrophages derived from *bcl-2*$^{+/+}$, *-2*$^{+/-}$, *and -2*$^{-/-}$ mice (Figure 6A) nor among macrophages derived from *bcl-2* transgenic mice (Figure 6B), showing specificity and implying that MDP activates other molecules (e.g., Nod2 or Cryopyrin) besides NALP1 that regulate signaling pathways leading to TNFα production. Note that preparations of murine macrophages varied in their sensitivity to MDP and sometimes required priming with a small amount of LPS as described (Sutterwala et al., 2006; Figure S12).

The differences in MDP-induced IL-1β production observed for *bcl-2* knockout and *bcl-2* transgenic mice correlated with increases and decreases, respectively, in proteolytic processing of caspase-1 (Figures 6C and 6D). Immunoblotting showed comparable levels of NALP1 protein in *bcl-2* transgenic and nontransgenic macrophages, excluding a trivial explanation (not shown). We therefore conclude that Bcl-2 restrains the MDP-induced activation of caspase-1 and secretion of the caspase-1 substrate IL-1β in primary cultured macrophages.

Bcl-2 Regulates MDP-Induced IL-1β Production In Vivo

We compared IL-1β production in wild-type versus *bcl-2* knockout mice injected with MDP. Prior to injection, no IL-1β was detectable in serum of *bcl-2*$^{-/-}$, *bcl-2*$^{+/-}$, or *bcl-2*$^{+/+}$ mice by ELISA (Figure 6E). At 3 hr after MDP injection, IL-1β serum levels rose to ~100 pg/mL in wild-type mice, declining substantially by 24 hr. In contrast, in *bcl-2* knockout mice, IL-1β serum levels were over 4-fold higher at 3 hr and remained persistently elevated at 24 hr (Figure 6E). In contrast to IL-1β, MDP-induced production of TNFα in vivo was not affected by *bcl-2* and was measured at 0, 3, and 24 hr postinjection with MDP (Figure 6E; data not shown). While a clear increase in MDP-induced IL-1β production was observed in *bcl-2* knockout mice, reproducible differences in IL-1β production were not detectable in the Bcl-2 transgenic mice (not shown), possibly because the transgene is expressed only in myeloid-lineage cells, whereas NALP1 is expressed in many tissues (Chu et al., 2001).

DISCUSSION

In *C. elegans*, it has been shown that the Bcl-2 ortholog CED-9 binds caspase-activator CED-4 and suppresses CED-3 protease activation (Metzstein et al., 1998). Heretofore, an analogous mechanism for controlling caspase activation has been lacking in higher organisms. We show that Bcl-2-family members Bcl-2 and Bcl-X_L interact with NALP1, blunting NALP1-mediated activation of proinflammatory caspases.

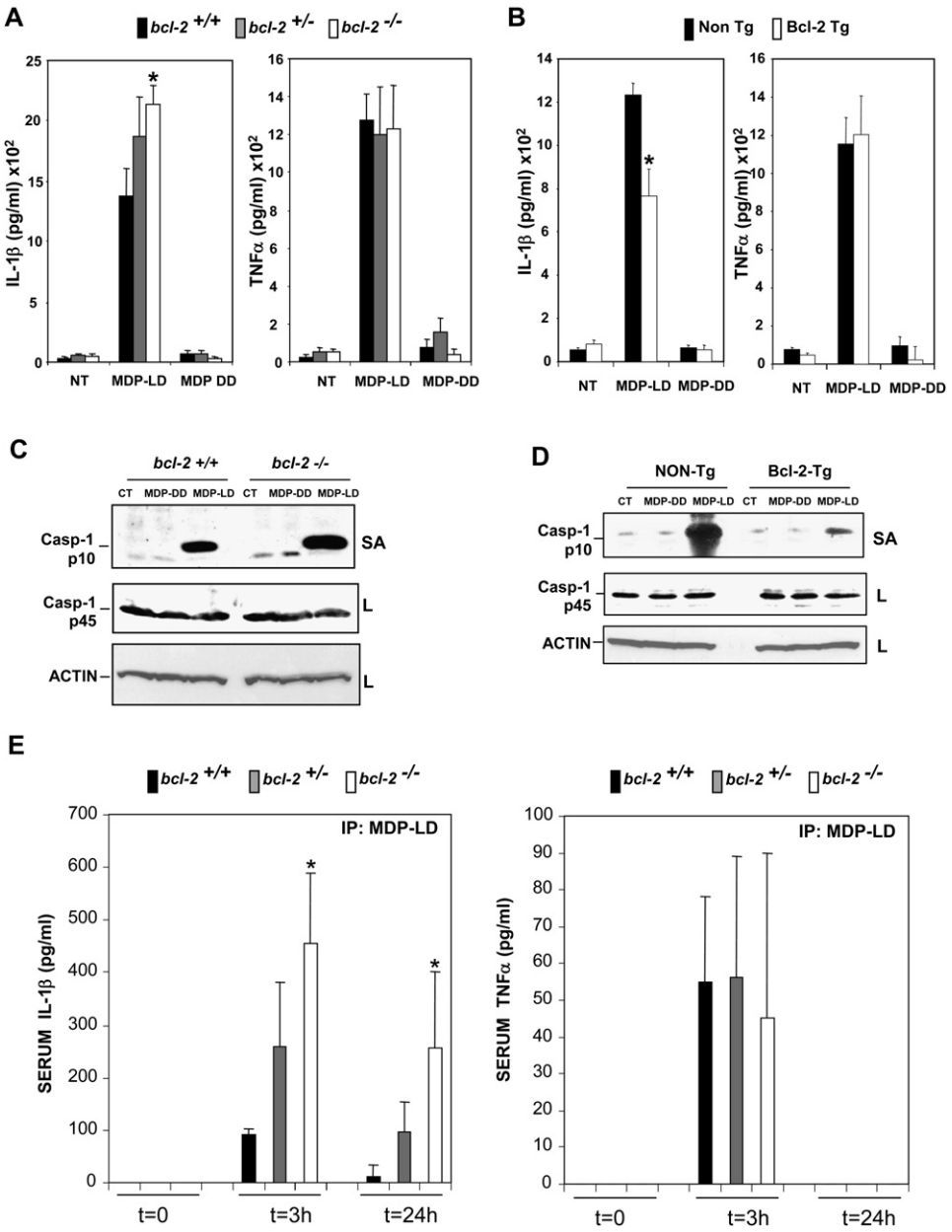

Figure 6. Bcl-2 Regulates MDP-Driven Caspase-1 Activation and IL-1β Production in Mice

(A and B) Bcl-2 regulates IL-1β production induced by MDP in cultured mouse macrophages. Macrophages from age-matched *bcl-2*$^{+/+}$, *-2*$^{+/-}$, and *-2*$^{-/-}$ mice (A) or from *bcl-2* transgenic (Tg) mice or control littermates (B; three mice per group) were stimulated with MDP-LD or MDP-DD for 2 hr, then pulsed with 2.5 mM ATP. After 2 hr, levels of IL-1β (left) or TNFα (right) released into culture supernatants were measured (mean ± SD ; n = 3). Asterisks denote $p < 0.05$ compared to wild-type cells.

(C and D) Bcl-2 regulates MDP-inducible caspase-1 activation in mouse macrophages. Macrophages from *bcl-2*$^{+/+}$ versus *bcl-2*$^{-/-}$ littermates and from *bcl-2*-Tg versus control littermates were incubated with biotinyl-VAD-fmk (30 μM) and subsequently stimulated with MDP-LD or MDP-DD for 2 hr. Lysates were analyzed as in Figure 5C.

(E) Bcl-2 regulates MDP-induced IL-1β production in vivo. Age-matched female *bcl-2*$^{+/+}$ (n = 5), *-2*$^{+/-}$ (n = 4), and *-2*$^{-/-}$ (n = 4) mice were injected i.p. with 100 ug/kg MDP. Serum was collected before (t_0) and at 3 hr and 24 hr after injection for measuring levels of IL-1β (left) and TNFα (right; mean ± SEM). Asterisks denote $p < 0.05$ compared to $^{+/+}$ control mice.

The human genome encodes >20 genes encoding NLR-family proteins (Kufer et al., 2005; Martinon and Tschopp, 2005). By analogy to structurally similar host-defense genes in plants, presumably the reason for expansion of this gene family is to provide diversity in recognition of pathogen-associated molecules through diversification

of the LRRs. It is interesting that the LRRs of NALP1 are required for Bcl-2/X_L binding, implying that the same domain used by NALP1 to recognize pathogen-associated MDP also binds Bcl-2/ X_L. The binding of Bcl-2 and ASC to NALP1, however, is unlikely to be directly competitive because ASC has been shown to interact with the PYRIN domain of NALP1, while the LRRs are necessary for Bcl-2/Bcl-X_L binding; thus, this suggests that these proteins recognize different conformational states of NALP1. Differences in the LRRs of NALP1 relative to other members of the NLR family may explain why Bcl-2 and Bcl-X_L bind NALP1 but not NALP2-4.

The loop regions of Bcl-2 and Bcl-X_L required for NALP1 binding are the least-conserved segments among the Bcl-2-family proteins, presumably explaining why Bcl-2 and Bcl-X_L, but not other Bcl-2-family proteins, bind NALP1. Since the loop region is subject to posttranslational modifications that modulate the antiapoptotic activity of Bcl-2 and Bcl-X_L, it will be interesting to explore the impact on NALP1 binding. The apparent utilization of the loop region by Bcl-2 and Bcl-X_L for engaging NALP1 differs structurally from the mechanisms used by CED-9 for binding CED-4 (Yan et al., 2005), implying that different means can be employed to accomplish the same goal. In this regard, profound structural differences have also been noted between orthologous human and *C. elegans* apoptosis regulators, such as CED-4 and its mammalian counterpart Apaf1 (Adams and Cory, 2002), which illustrates how basic paradigms for function are preserved despite structural diversification during evolution. However, it should be noted that the loop domains of Bcl-2 and Bcl-X_L may be necessary to generate conformational states competent to bind NALP1 rather than serving directly as ligands for binding NALP1.

The data presented here demonstrate an apoptosis-independent phenotype for Bcl-2 and Bcl-X_L. However, while the proinflammatory branch of the caspase family (caspases-1,4,5 in humans) that NALP1 regulates is principally involved in cytokine activation, these proteases have also been implicated in apoptosis induction in a variety of pathological contexts, including infection of macrophages by bacteria and neuronal cell death induced by ischemia (Navarre and Zychlinsky, 2000; Zhang et al., 2003). Thus, the ability of Bcl-2 and Bcl-X_L to suppress an inflammatory caspase-activating NLR-family member (NALP1) may provide an additional mechanism for cell preservation during stress (Figure S13). Additional links between NLR-family proteins and the core components of the apoptosis machinery have been reported that may also be relevant. For example, ASC has been reported to bind Bax, collaborating in apoptosis induction (Ohtsuka et al., 2004). Moreover, NALP1 (NAC) can associate with Apaf-1 (Chu et al., 2001; Figure S6), an activator of apoptotic caspases. Thus, an intricate network of protein interactions appears to exist that involves components of the innate immunity and apoptosis machineries, presumably allowing for coordination of cell death and host defense. A prediction of these findings is that some viral homologs of Bcl-2 will be found to interact with and inhibit NLR-family members as a mechanism of blunting host-defense while simultaneously suppressing cell death for purposes of preserving hosts for viral replication.

EXPERIMENTAL PROCEDURES

Additional details for methods are provided in the Supplemental Data.

Production of GST Proteins

GST-fusion proteins containing Bcl-X_L, Bcl-2, Bcl-W, Bcl-B, Bfl-1, and Mcl-1 were expressed from pGEX 4T-1 (Zhai et al., 2006). All constructs were engineered with a stop codon to exclude the C-terminal transmembrane (TM) domains. GST-fusion proteins corresponding to Bcl-2-family proteins were confirmed to be properly folded based on ability to bind fluorochrome-conjugated BH3 peptides with submicromolar affinity as shown by fluorescence polarization assays (Zhai et al., 2006).

Cell Lines and Transfections

HEK293T and HeLa cells were cultured in DMEM high-glucose medium (Irvine Scientific), while THP-1 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS). Transfection of HEK293T and HeLa cells was performed using Lipofectamine 2000 reagent (Invitrogen). THP-1 cells were electroporated using an instrument from AMAXA (see below). THP-1 cells were differentiated into macrophages by culturing overnight with 50 ng/ml TPA (Calbiochem).

THP-1 Cell Transfection with siRNA

siRNAs were purchased from AMBION (Table S1). Various double-stranded ribo-oligonucleotides (dsRNA) with overhanging 3′ deoxy TT were prepared that target NALP1, Bcl-2, Bcl-X_L, Bcl-W, or Bcl-B mRNAs. Control dsRNAs constituting scrambled versions of NALP1 siRNA sequences were also tested (Table S1).

THP-1 cells were electroporated using AMAXA system (Nucleofector), suspending 2 ×1 0^6 cells in 100 μl of solution V (AMAXA) containing 2 μg siRNA or dsRNA control, and applying an electrical discharge using program T12. The efficiency of transfection (80%) was monitored using a fluorescence isothiocyanate (FITC)-conjugated control dsRNA (Ambion). Cells were cultured for 56 hr then differentiated by culturing with 50 ng/mL TPA for 24 hr. Adherent macrophages were then stimulated for 4 hr with 1 μg/ml LPS or 2 μg MDP-LD, followed by 2.5 mM ATP for 20 min. Culture supernatants were assayed for IL-1β after 2 hr. Culture supernatants and cell extracts were also analyzed by immunoblotting in some cases.

Lentivirus Infections

The lentiviral construct PRRL.Sin18.PGK-hBCL2.ppt was produced as described (Zufferey et al., 1998). THP-1 cells were transduced with the virus at a MOI = 15 in suspension with 10 μg/ml polybrene. At 3 days postinfection, infected cells were serially diluted to achieve single cells per well in 96-well plates. Bcl-2 expression in 19 of the 40 recovered clones was confirmed by immunofluorescence using FITC-conjugated anti-Bcl-2 antibody. Two independent clones were further amplified and used for subsequent experiments.

Murine Macrophage Cultures

Bone marrow cells were harvested from leg bones of sacrificed 5-week-old *bcl-2* transgenic, *bcl-2* knockout, and littermate control mice on a C57Bl/6 background (Domen et al., 2000; Wang et al., 2005). Bone marrow cells were cultured at 37°C with 5% CO_2 in RPMI 1640 medium with 10% FBS. CSF-1 (SIGMA) was added to medium for 1 week to promote differentiation. Adherent macrophages in 24-well plates were stimulated for 3 hr with various amounts of LPS, MDP-LD, or MDP-DD then cultured for a further 20 min in fresh

medium containing 2.5 mM ATP, washed, and cultured in 0.5 ml fresh medium for 2 hr before collecting culture supernatants for IL-β assay.

Caspase Activity

For assessing effects of Bcl-2-family proteins on caspase-1 activation, cytosolic extracts were prepared from differentiated THP-1 cells before or after treatment with LPS or MDP (Bruey et al., 2000) using lysis buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 0.5% NP-40, and 0.1 mM Na_3VO_4. THP-1 cell extracts (10 μg) were mixed with 30 μg of extract from NALP1-expressing HEK293T cells in a total volume of 0.1 mL, then 1–5 μg of recombinant GST proteins were added, and the mixtures were incubated at 37°C for 45 min before adding caspase substrate, Ac-WEHD-AFC.

To recover active caspases from cells, differentiated THP-1 macrophages were incubated with biotinyl-VAD-fmk (30 μM; Alexis) 0.5 hr before stimulation with 2 μg/ml MDP-LD or MDP-DD for 3 hr. Cells were then resuspended in "lysis solution" (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 0.3% NP-40, 0.1 mM Na_3VO_4, 20 $\mu g/ml^{-1}$ leupeptin, 20 $\mu g/ml^{-1}$ aprotinin, and 1 mM PMSF), and active caspases were recovered using Streptavidin-Sepharose (SIGMA), adding 30 μl of 1:1 Streptavidin-Sepharose suspension per 250 μl of lysate. Beads were washed in the lysis buffer and analyzed by SDS-PAGE/immunoblotting using an antibody that detects the p10 small subunit of processed human caspase-1 (Santa Cruz, CA).

IPs and Immunoblotting

Immunoblotting was performed as described previously (Guo et al., 2003). For IPs, 10^8 THP-1 cells were used. For coIPs, 2×10^6 HEK293T cells were cultured in 50 μM benzyloxycarbonyl-Val-Ala-Asp (O-methyl)-fluoromethyl ketone (zVAD-fmk; Enzyme Systems Products) to prevent apoptosis. Cells were suspended in 0.5mL lysis solution, cleared by incubation with 15 μl protein G Sepharose 4B (Zymed), and then incubated with 15 μl of either polyclonal antibody or rabbit IgG control serum (Zymed; San Francisco) and 40 μl protein G at 4°C overnight. Samples were then washed four times with lysis buffer, boiled in Laemmli buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE)/immunoblotting. Alternatively, lysates were directly analyzed by immunoblotting after normalization for total protein content.

Subcellular Fractionation

Stepwise extraction of cytosolic fractions and organelle/membrane fractions was performed using ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) and 2×10^6 THP-1 macrophages.

Animal Experiments

A cohort of mice (8 to 10 weeks old) was injected intraperitoneally with 100 μg/kg-1 MDP-LD in PBS. For serum cytokine measurements, animals were bled retro-orbitally before or at various times after MDP injection.

Statistical Analysis

Most data were presented as the mean ± SD from at least three independent experiments. Statistical comparisons between different treatments were performed by unpaired Student's t test and $p \leq 0.05$ was considered statistically significant.

Supplemental Data

Supplemental Data include one table, 13 figures, Experimental Procedures, and References and can be found with this article online at http://www.cell.com/cgi/content/full/129/1/45/DC1/.

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